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Reliability of High-Content Disks and Modified Broth Dilution Tests for Detecting Staphylococcal Resistance to the Penicillinase-Resistant Penicillins

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In vitro susceptibility tests were performed with 271 isolates of *Staphylococcus* species (204 *Staphylococcus aureus*), including 110 strains resistant to the penicillinase-resistant penicillins. Disks containing 5 or 10 μg of methicillin, 1 or 4 μg of oxacillin, and 1 or 4 μg of nafcillin were evaluated. After a full 24 h of incubation at 35°C, tests with 1-μg oxacillin disks provided optimal results. Use of the more potent oxacillin, nafcillin, or methicillin disks only increased the number of false-susceptible test results. For broth microdilution tests, 2% NaCl should be added to cation-supplemented Mueller-Hinton broth, and MICs should be recorded after a full 24 h at 35°C. Microdilution tests with oxacillin in broth with 2% NaCl were more reliable than similar tests with methicillin.

The antistaphylococcal activity of most penicillins is seriously compromised by the fact that the majority of strains produce β-lactamase enzymes which are capable of inactivating the drugs before they can act. This problem was resolved by developing a series of penicillins which are relatively resistant to staphylococcal β-lactamases. Methicillin was the first of the series of drugs known as penicillinase-resistant penicillins (PRPs). In the United States, an increased prevalence of PRP-resistant staphylococci in some institutions has created a serious clinical problem, and accurate methods for detecting such strains are needed.

Some staphylococci are resistant by virtue of the fact that they produce an unusually large amount of β-lactamase, which can slowly inactivate methicillin and other PRPs (10). Resistance by other mechanisms is characteristically a heteroresistance, i.e., within the same culture, some cells are susceptible but other cells are resistant (7, 13). The proportion of PRP-resistant cells within a broth culture may vary from strain to strain, but the susceptible cells often predominate. Under standard testing conditions, the resistant population may not be detected since the susceptible cells grow much more readily. If the inoculum is prepared by preincubating it for several hours in a broth medium, the susceptible cells might overgrow the resistant portion of the cell population. Consequently, for testing staphylococci, the inoculum should be prepared as a direct suspension of freshly isolated colonies rather than as a log-phase broth culture (11, 12). In vitro test conditions can be altered to increase the opportunity to detect growth of the PRP-resistant cell populations. Incubation at lower temperatures (25 to 30°C), prolonged incubation (48 h), and use of media with increased salt content have been recommended (1, 4, 7, 13–15).

In 1977, Barry and Badal (2) recommended the addition of 5% NaCl to Mueller-Hinton broth used for microdilution susceptibility tests with methicillin, oxacillin, or nafcillin. In 1983, Thornsberry and McDougal (15) confirmed these observations. Because they used Mueller-Hinton broth with added $CaCl_2$ and $MgCl_2$, they recommended use of 2% NaCl rather than 5% NaCl. In both of the above studies, a full 48 h of incubation was required to detect PRP resistance in unsupplemented broth, but with added NaCl, the vast majority of PRP-resistant strains were properly identified after overnight incubation.

In 1972, Drew et al. (6) concluded that the standard disk test was satisfactory for identifying most PRP-resistant staphylococci. They recommended the use of an oxacillin disk because methicillin was less stable during storage. In 1973, Thornsberry et al. (14) confirmed those observations and also found that the incubation temperature should not exceed 35°C. In 1984, Boyce (3) demonstrated that the disk test must be incubated at 35°C for a full 24 h, rather than the usual 16 to 18 h. He also recommended use of a 1-μg oxacillin disk in preference to the 5-μg methicillin disk. In all reported studies, authors describe a zone of inhibition with small inner colonies which may or may not be detected under standard test conditions. Careful observation of the test plates will often reveal tiny inner colonies that are easily overlooked when viewed from the back of the plate against a black background (12).

Recently, McDougal and Thornsberry (9) suggested that the use of more potent PRP disks, with appropriately adjusted interpretive criteria, may improve the accuracy of the test. The present report evaluates the relative accuracy of high- and low-content disks. We also confirm the importance of adding NaCl to the broth medium for microdilution tests with methicillin and oxacillin.

MATERIALS AND METHODS

Bacteria. Isolates were selected from our stock culture collection and from the Centers for Disease Control (Atlanta, Ga.). These strains represent clinical isolates from a broad sample of geographically separate institutions. The majority of isolates were collected from institutions within the United States over the past 3 to 4 years. The collection included 204 *Staphylococcus aureus* and 67 coagulase-negative *Staphylococcus* species; 110 strains were resistant to one or more of the PRPs.

Susceptibility tests. Inocula were prepared by selecting colonies from an overnight blood agar plate. A saline suspension of colonies was adjusted to match the turbidity of a

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TABLE 1. Methicillin and oxacillin microdilution susceptibility test results in cation-supplemented Mueller-Hinton broth with or without 2% NaCl added, read after 24 h at 35°C

24-h MIC (μg/ml)	No. of isolates in each MIC category[a]							
	Methicillin				Oxacillin			
	Without NaCl		With 2% NaCl		Without NaCl		With 2% NaCl	
	PRPs[b]	PRPr[c]	PRPs	PRPr	PRPs	PRPr	PRPs	PRPr
>16		53		89		51		89
16		16		9		17		12
8.0		16		8		17		2
4.0	5	9	15	1		15		4
2.0	51	15	80	3		7	2	1
1.0	102	1	60		1	2	14	1
0.5	1		5		25	1	20	1
≤0.25	2		1		135		125	

[a] Horizontal lines designate the interpretive breakpoints recommended by Thornsberry and McDougal (15) for tests with added NaCl.
[b] PRPs, PRP-susceptible isolates (susceptible by all methods evaluated), including 127 *S. aureus* and 34 coagulase-negative staphylococci.
[c] PRPr, PRP-resistant isolates (resistant by one or more microdilution tests), including 77 *S. aureus* and 33 coagulase-negative staphylococci.

McFarland 0.5 standard, and this suspension was used to simultaneously inoculate microdilution trays and disk tests.

For microdilution susceptibility tests, Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) was supplemented with calcium and magnesium cations, as described by the National Committee for Clinical Laboratory Standards (NCCLS) (11). Doubling dilutions of methicillin (0.25 to 32 μg/ml) and of oxacillin (0.12 to 16 μg/ml) were prepared in the broth with and without additional NaCl (2%, wt/vol). The microdilution trays were stored at −40°C until needed. The inoculum density was approximately 5×10^5 CFU/ml, and MICs were recorded after 24 h of incubation at 35°C in ambient air. The majority of strains (25 of 29) with discrepancies between methicillin and oxacillin MIC categories were reincubated, and MICs were again recorded after a total of 48 h.

Disk susceptibility tests were performed by the NCCLS procedure (12). Disks containing methicillin (5 and 10 μg), oxacillin (1 and 4 μg), or nafcillin (1 and 4 μg) were produced by Difco Laboratories. The zones of inhibition were measured to the nearest whole millimeter. When there was a definite zone with visible colonies throughout that zone, the organism was considered to be resistant (no zone).

RESULTS

The distribution of 24-h MICs, with and without added NaCl, is displayed in Table 1. When all six disks and all four MICs indicated susceptibility, the strain was designated as being PRP susceptible. That category included 127 *S. aureus* strains and 34 coagulase-negative staphylococci. The remaining strains were designated PRP resistant since one or more of the MICs were ≥8.0 μg/ml (methicillin) or ≥4.0 μg/ml (oxacillin). Intermediate MICs of 8.0 μg of methicillin per ml or 4.0 μg of oxacillin per ml were observed only with strains that were classified as being PRP resistant by other tests. Consequently, intermediate MIC categories do not seem to be applicable and were not used in subsequent analyses. Without NaCl, PRP-susceptible strains were all inhibited by ≤1.0 μg of oxacillin per ml and MICs for only three resistant strains were ≤1.0 μg/ml. When NaCl was added, the appropriate oxacillin-susceptible breakpoint was raised to ≤2.0 μg/ml. When testing methicillin, a susceptible MIC breakpoint of ≤4.0 μg/ml was appropriate, with or without added NaCl.

One or more of the four microdilution tests (methicillin and oxacillin, each with and without added NaCl) failed to detect resistance among 29 of the 110 PRP-resistant staphylococci after 24 h of incubation. Most of those 29 strains were reexamined after 48 h of incubation, and the results of all observations are recorded in Table 2. With additional NaCl, methicillin tests failed to identify 4 of the 29 PRP-resistant strains after 24 h, and one of those strains was resistant after 48 h. With added NaCl and 48 h of incubation, all resistant strains were resistant to oxacillin, but three strains were misidentified after 24 h. Only one strain (SA2) would have been misidentified at 24 h if both drugs were tested and if resistance to either drug was considered evidence of PRP resistance. The few strains that would have been misidentified by 24-h broth microdilution tests were confirmed to be PRP resistant by tests with 1-μg oxacillin disks.

Table 3 summarizes a comparison of low- and high-content disk tests. The 5-μg methicillin disk misidentified 15 of 110 PRP-resistant strains, and the 10-μg methicillin disk misidentified 10 additional strains. Both 1- and 4-μg nafcillin disks were also less reliable. The 1-μg oxacillin disk provided the fewest interpretive errors (one false-resistant and one false-susceptible). Five additional errors were recorded when the oxacillin disk content was increased to 4 μg. The interpretive discrepancies with 1-μg oxacillin disks could have been reduced to minor errors if the zone-size breakpoints for tests with 5-μg methicillin disks (≤9 mm and ≥14 mm) were used. That would result in a larger proportion of staphylococci (4.8% of our strains) that give intermediate or indeterminant disk test results. With the presently recommended zone-size criteria (≤10 mm and ≥13 mm), only 1.8% of our strains gave indeterminant disk test results.

DISCUSSION

Definition of an optimal, but yet practical method for absolute detection of all staphylococcal resistance to the penicillinase-resistant penicillins seems impossible. Although the majority of PRP-resistant strains are readily detected with standard susceptibility test methods, some strains are much more difficult to identify. Cultures with β-lactamase-mediated PRP resistance often have MICs near

TABLE 2. In vitro test data with 29 PRP-resistant staphylococci that would have been misidentified with at least one microdilution test systems[a]

Strain no.[b]	Methicillin MICs (μg/ml)[c]				Oxacillin MICs (μg/ml)[c]			
	No NaCl		2% NaCl		No NaCl		2% NaCl	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
SA1	1.0	4.0	2.0	4.0	2.0	>16	16	>16
SA2	2.0	2.0	2.0	2.0	0.5	2.0	0.5	16
SA3	2.0	2.0	4.0	32	16	>16	>16	>16
SA4	2.0	2.0	2.0	8.0	4.0	4.0	16	>16
SA5	2.0	2.0	8.0	8.0	4.0	>16	>16	>16
SA6	2.0	2.0	32	32	>16	>16	>16	>16
SA7	2.0	4.0	32	32	4.0	16	>16	>16
SA8	2.0	8.0	16	>32	8.0	16	>16	>16
SA9	2.0	32	32	32	16	>16	>16	>16
SA10	2.0	>32	32	>32	>16	>16	>16	>16
SA11	4.0	4.0	8.0	8.0	2.0	4.0	4.0	8.0
SA12	4.0	4.0	16	16	2.0	4.0	8.0	8.0
SA13	4.0	8.0	16	16	8.0	16	>16	>16
SA14	4.0	8.0	16	32	8.0	8.0	16	>16
SA15	4.0	32	32	32	8.0	>16	>16	>16
SA16	8.0	32	>32	>32	2.0	8.0	16	16
CN1	2.0	4.0	8.0	16	2.0	4.0	2.0	4.0
CN2	2.0	32	>32	>32	4.0	>16	>16	>16
CN3	2.0	—[d]	32	—	4.0	—	16	—
CN4	2.0	—	>32	—	8.0	—	>16	—
CN5	2.0	>32	32	>32	8.0	>16	16	>16
CN6	2.0	>32	>32	>32	8.0	>16	>16	>16
CN7	4.0	4.0	8.0	32	2.0	>16	4.0	>16
CN8	4.0	32	>32	>32	8.0	>16	>16	>16
CN9	4.0	>32	>32	>32	4.0	>16	>16	>16
CN10	4.0	—	>32	—	>16	—	>16	—
CN11	8.0	8.0	8.0	8.0	1.0	4.0	1.0	4.0
CN12	8.0	—	>32	—	1.0	—	8.0	—
CN13	8.0	8.0	8.0	8.0	2.0	2.0	4.0	4.0

[a] 81 other isolates were resistant by all four dilution tests in the first 24 h of incubation.
[b] *S. aureus* (SA) or coagulase-negative staphylococci (CN).
[c] Microdilution tests performed in cation-supplemented Mueller-Hinton broth with or without 2% NaCl added; MICs read after 24 h and again after 48 h at 35°C.
[d] —, 48-h observations were not recorded.

the breakpoint separating susceptible and resistant populations (10). If such strains are tested on different days, they might be miscategorized from time to time because of the normal variations in inoculum size and other variables which can cause minor inconsistencies. We did not attempt to identify the proportion of strains with this resistance mechanism compared with strains with true heteroresistance in our culture collection. Heteroresistant cultures with a relatively low proportion of resistant cells are also easily misidentified unless special precautions are taken. The addition of 2% NaCl to cation-supplemented Mueller-Hinton broth for microdilution tests and prolonged incubation at 35°C are two such precautions that can be taken to improve the reliability of the standardized tests.

For performing microdilution tests, this report confirms previous observations (2, 15) that the broth medium should be supplemented with additional NaCl. With the addition of 2% NaCl to cation-supplemented Mueller-Hinton broth, oxacillin detected PRP resistance among 107 of 110 (97%) resistant strains after 24 h of incubation and all strains were resistant after 48 h. Two of the three strains that were misidentified by tests with oxacillin after 24 h were resistant to methicillin on day 1. Both drugs could be tested routinely (with added NaCl) to increase the reliability of 24-hour MICs. Resistance to one or both drugs should be interpreted to represent resistance to all PRPs.

In this study we operated under the assumption that resistance to one PRP is accompanied by cross-resistance to other drugs in that family. Among the isolates included in this study, we found no evidence to the contrary. However, false-susceptible in vitro tests can be misleading, since some strains may initially appear to be susceptible to one drug but resistant to another. In those cases, inapparent resistance is usually detected with prolonged incubation or by the addition of NaCl to the broth medium. Some coagulase-negative staphylococci might be truly susceptible to one PRP but resistant to others (5). No such strains could be identified in the current series of tests with stock cultures from a wide variety of institutions.

For microdilution tests, we recommend that oxacillin should be prepared in cation-supplemented Mueller-Hinton broth with an additional 2% NaCl and that MICs should be recorded after a full 24 h of incubation at 35°C. Similar tests with methicillin may be performed at the same time to increase the reliability of 24-h observations. Under these test conditions, strains for which the oxacillin MIC is ≥4.0 μg/ml or the methicillin MIC is ≥8.0 μg/ml may be considered PRP resistant. Very few truly resistant strains should appear to be susceptible to both drugs after 24 h. Since the error rate associated with 24-h test results is quite low, routine reincubation of all staphylococcal susceptibility tests does not appear to be a cost effective procedure unless one is dealing with a unique endemic situation. It is important to emphasize the fact that our conclusions are based on data accumulated with stock cultures. The character of the strains that are endemic within an institution should influ-

TABLE 3. Distribution of zone diameters with 161 PRP-susceptible (S) and 110 PRP-resistant (R) isolates of *Staphylococcus* spp.

Zone diam (mm)	No. of strains with the designated zone diam[a]											
	Methicillin				Oxacillin				Nafcillin			
	5 μg		10 μg		1 μg		4 μg		1 μg		4 μg	
	S	R	S	R	S	R	S	R	S	R	S	R
6		88		72		106		99		98		79
7				1								1
8						1						1
9				1						1		2
10	1	2		2	1			1	1	1		2
11		3		1		2				2		
12	1	1		2	3			1				1
13	1	1		2	6	1		1	8			
14	6	4		4	4			2	6			
15	5	1		3	2							3
16	4	2		6	4			1	6			2
17	5	3	1	4	4				9			4
18	10		1		9		1	1	22			2
19	21	3	4	3	15		1	1	23	2	1	2
≥20	107	2	155	9	113		159	3	86	6	160	11
False-resistant[b]	0		0		1		0		1		0	
False-susceptible[c]	15		25		1		6		8		21	

[a] All determinations made after 24 h at 35°C. Horizontal lines designate interpretive breakpoints proposed by McDougal and Thornsberry (9) or recommended by the National Committee for Clinical Laboratory Standards (12).
[b] Number of susceptible strains reported to be resistant by the disk test.
[c] Number of PRP-resistant strains reported to be susceptible by the disk test.

ence the selection of a testing system which is optimal for use in that institution.

For disk susceptibility tests, the plates should be incubated for a full 24 h at temperatures that do not exceed 35°C (3, 14). The zones of inhibition must be carefully examined for evidence of resistant colonies which often appear as small pinpoint colonies scattered throughout an otherwise clear zone of inhibition. Other growth patterns have been described (9). The character of the inner growth may change with different Mueller-Hinton agars (8). Such inner growth is often easily overlooked, but may become much more visible with prolonged incubation. Resistance was more accurately detected with the 1-μg oxacillin disk than with any of the other PRP disks tested. For testing coagulase-negative staphylococci, Coudron et al. (5) recommended use of a 5-μg methicillin disk rather than a 1-μg oxacillin disk since they encountered strains which appeared to be methicillin resistant but oxacillin susceptible. They also concluded that 10-μg methicillin disks were not as reliable as 5-μg methicillin disks.

McDougal and Thornsberry (9) have recommended the use of more potent disks, i.e., 10 μg rather than 5 μg of methicillin and 4 μg rather than 1 μg of oxacillin or nafcillin. The data that are described in this report failed to confirm the need for increasing the disk potency. Using commercially prepared disks for all three drugs, the more potent disks were actually less reliable than the currently recommended disks. The greatest accuracy was obtained when tests were performed with 1-μg oxacillin disks; i.e., after 24 h, we observed only one false-resistant (0.4%) and one false-susceptible (0.4%) result. Only five (1.8%) strains gave intermediate zones of 11 or 12 mm: that included two resistant strains and three susceptible strains. The low error rate could be improved if the interpretive zone-size standards were modified to ≤9 mm and ≥14 mm, but that would provide too many tests with indeterminant results.

In summary, the standardized disk susceptibility test with 1-μg oxacillin disks is reasonably reliable for detecting staphylococcal resistance, provided that the plates are allowed to incubate for a full 24 h at 35°C and that the zones are carefully examined to detect small inner colonies. Use of 4-μg oxacillin disks or 10-μg methicillin disks only increased the number of false-susceptible disk test results. For microdilution tests, oxacillin should be diluted in cation-supplemented Mueller-Hinton broth with 2% NaCl added and MICs should be recorded after a full 24 h of incubation at 35°C. Routine tests of both methicillin and oxacillin could reduce the number of interpretive errors that might be expected after 24 h of incubation.

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